Effect of Continuous-Wave Low-Intensity Ultrasound in Inflammatory Resolution of Arthritis-Associated Synovitis

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Background. Low-intensity ultrasound (LIUS) can reduce pain and improve function in arthritic joints. Neutrophils are first-line actors in host defense that recruit macrophages. Dead neutrophils are removed during resolution of inflammation. Delayed neutrophil clearance can lead to extended inflammation or even chronic autoimmune disease. Although neutrophil extracellular traps (NETs) in arthritic tissue are involved in the pathogenesis of arthritis, their functional role has not been clarified.

Objectives. This study aimed to investigate the effect of LIUS on synovial inflammation and its resolution via neutrophil clearance.

Methods. Synovitis was induced by intra-articular injection of complete Freund’s adjuvant (CFA) into the left knee joint of 58 adult male Sprague-Dawley rats. Low-intensity ultrasound (1 MHz, 200 mW/cm²) was applied for 10 minutes daily. Neutrophil clearance was assessed with the expression of myeloperoxidase (MPO). In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and NET formation in the synovium were observed. In neutrophil and macrophage cultures from peripheral blood, the effect of NET clearance by LIUS was investigated.

Results. In CFA-induced synovitis, MPO-positive neutrophils peaked after 2 to 3 days, filling the inflammatory core. Monocytes and macrophages in the periphery later infiltrated the core and were reduced thereafter. Low-intensity ultrasound reduced synovial hyperplasia and induced earlier MPO clearance. Neutrophils in the core of the inflamed synovium exhibited NET formation, which LIUS increased. Low-intensity also induced NETs in peripheral polymorphonuclear cells in an intensity-dependent manner and potentiated phorbol myristate acetate (PMA)-induced NETosis. The PMA-induced NETs were cleared by macrophages; clearance was enhanced by LIUS.

Limitations. The effect of LIUS on CFA-induced inflammation was observed only during the acute phase. Although the effect of LIUS on NETosis in the in vitro neutrophil culture system was clear, the in vivo NETosis cannot be quantified.

Conclusions. Neutrophil extracellular traps act in inflammatory synovitis, and LIUS enhanced the NETs and resulted in neutrophil clearance by enhancing the phagocytosis of macrophages, which might be a factor underlying the therapeutic effect of LIUS in arthritic synovium.
Articulitis, such as osteoarthritis (OA) and rheumatoid arthritis (RA), are chronic and progressive joint inflammatory diseases with remission and aggravation. The early introduction of disease-modifying therapy inhibits its progressive structural change.\(^1\) Low-intensity ultrasound (LIUS) is often used as adjunct therapy for the symptomatic treatment of arthritis. Low-intensity ultrasound reduces pain and improves physical function in patients with knee OA or RA.\(^3\)-\(^9\) Combining LIUS with beta-methasone, diclofenac, or hyaluronan enhances their anti-inflammatory effect, analgesic effect, or increased drug absorption.\(^10\)-\(^12\) Low-intensity ultrasound demonstrates significant cartilage protection in in vitro and in vivo models of OA.\(^13\),\(^14\) It also reduces knee edema and inflammatory cells in an adjuvant-induced synovitis model.\(^15\) in contrast, LIUS increases vascular permeability and mast cell degranulation.\(^16\) Therefore, the mechanism underlying the protective effect of LIUS on inflammatory events must be investigated further.

Diverse innate inflammatory processes, including the infiltration of neutrophils and macrophages into joint tissue, and elevated levels of inflammatory cytokines are involved in the pathogenesis of arthritis.\(^17\)–\(^19\) Synovitis is increasingly recognized as an important feature of arthritic pathophysiology.\(^20\) The infiltration of innate immune cells, such as macrophages and polymorphonuclear (PMN) cells, into the synovial membrane reflects global disease activity in arthritis.\(^21\) These immune cells can produce cytokines that affect the activation of resident synovial fibroblasts and the chemotaxis of monocytes through blood vessels, increase angiogenesis, increase the destruction of cartilage and bone, or reduce regenerative mediators.\(^22\) Therefore, the modulation of infiltrated innate immune cells in synovial tissues can be a potent therapeutic target in the management of OA.

Neutrophils have a short life span in host tissue and can kill invaded bacteria or microorganisms with oxidative intermediates and granule-packaged proteolytic enzymes.\(^23\) During resolution, the regulation of macrophages’ capacity to clear neutrophils is an important factor that determines whether inflammation ultimately resolves or progresses to chronic arthritis.\(^24\) There are important issues regarding how the first invading neutrophils can be cleared, how monocytes mobilize into synovial tissue, and how phagocytosis of neutrophils can be regulated. Delay of apoptosis is one important mechanism underlying neutrophil clearance during the resolution phase of inflammation is now garnering attention.

We reported previously that LIUS significantly reduces arthritic knee edema, as well as the accumulation of MPO-positive neutrophils and the infiltration of ionized calcium binding adaptor molecule 1 (Iba1)-positive monocytes and phagocytes into synovial tissue in complete Freund’s adjuvant (CFA)-induced arthritis.\(^15\) In the present study, we investigated whether NETosis lead to autoimmune diseases, including systemic lupus erythematosus and RA.\(^3\)-\(^5\) However, the role of NETs in neutrophil clearance during the resolution phase of inflammation is now garnering attention.

Exposure to LIUS

A LIUS generator with 6 plane disk transducers (18-mm diameter) resonating at a frequency of 1 MHz was made (HNT Medical). Low-intensity ultrasound in continuous-wave mode was applied for 10 minutes daily for 1 to 4 days. The ultrasound transducers were placed under the table with apertures. To apply LIUS in vivo, the rats were tightly held with a special holder during LIUS intervention, and the LIUS probe was attached to the anterior left knee joint, where ultrasound transmission gel (Sani-pia, Ilsan, Korea) was applied to minimize reflection (eFigure, image 1A, available at ptjournal.apta.org). Afterward, the animals were euthanized by perfusion to collect tissues. To apply LIUS in vitro, a culture dish filled with medium was placed in the LIUS field at a distance of less than 4 mm, and the gap between the culture dish and the transducer was filled with ultrasound transmission gel (eFigure, image 1B, available at ptjournal.apta.org).

Immunohistochemistry

The rats were anesthetized with sodium pentobarbital and euthanized by transcardial perfusion with saline followed by 4% paraformaldehyde (PFA, Sigma-Aldrich) in phosphate-buffered saline (PBS). The synovium was removed immediately from the knee joint, post-fixed overnight in 4% PFA and subse-
quently processed for immunohistochemistry. The sectioned synovial tissue was blocked with 5% normal goat serum and then incubated with rabbit anti-MPO (Dako, Glostrup, Denmark), anti-Iba1 (Wako Pure Chemical Industries, Richmond, Virginia), anti-neutrophil elastase (NE, Abcam, Cambridge, United Kingdom), and anti-histone (Cell Signaling Technology, Danvers, Massachusetts) primary antibodies, followed by incubation with secondary antibody labeled with Alexa Fluor 488 or 594 (Invitrogen Corp, Eugene, Oregon). The NET DNA was stained with DRAQ5 (Invitrogen Corp), a cell-permeable DNA dye, and nuclei were stained with Hoechst33258 (Sigma-Aldrich). Tissues were mounted using VECTASHIELD (Vector Lab, Burlingame, California), and images were acquired with a Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena, Germany).

TUNEL Assay
Apoptotic cell death was detected by DNA fragmentation using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL-positive cells were stained with TUNEL POD (Roche, Indianapolis, Indiana) according to the manufacturer’s directions. Briefly, sectioned synovial tissues or cultured neutrophils were fixed with 4% PFA and then blocked with 3% H2O2 in methanol. The blocked tissues or cells were permeabilized with 0.1% Triton X-100 in PBS and then incubated with TUNEL-label solution for 1 hour. The unbiased stereological estimation of TUNEL-labeled cells in the core of neutrophils was made using optical fractionator. The sections used for counting covered the entire synovium was selected with 8 to 9 sections in a series. A counting frame was placed randomly. Images were acquired with an LSM 710 confocal microscope.

Western Blot Analysis
Synovial tissues were collected at 2 to 5 days after CFA injection and homogenized to prepare proteins for Western blot analysis as described previously. Briefly, 50 μg of each protein sample was separated on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, Massachusetts) with a semi-dry transfer cell (BIO-RAD, Hercules, California). The membranes were incubated overnight with antibodies against MPO, Iba1, or β-actin (Sigma-Aldrich). To quantitify chemiluminescent signals, PVDF membranes were scanned with a Fuji Film LAS-1000 system (Fujifilm Corp, Tokyo, Japan) and analyzed using Multi Gauge software (version 3.0) (Fujifilm Corp).

Isolation and Culture of Primary Rat Neutrophils and Differentiation of Monocyte-Derived Macrophages
Polymorphonuclear cells and peripheral blood mononuclear cells (PBMCs) were isolated from rat arterial blood through density gradient centrifugation using Ficoll-Plaque Plus (GE, Stockholm, Sweden) according to a protocol from the manufacturer. To prepare neutrophil-enriched culture, PMN cells were further isolated from the erythrocyte fraction by hypotonic lysis. Neutrophils were resuspended in RPMI 1640 media (Gibco, Grand Island, New York) supplemented with 0.1% penicillin/streptomycin, 10 mM L-glutamine, and 10 mM pyruvate, and the cells were plated at a density of 1 × 10⁶ cells/mL. Neutrophil elastase and F4/80 (Santa Cruz, Dallas, Texas) staining of PMN cells were used to assess the purity of the cell population. The PMN fraction consisted of >99% neutrophils. To prepare monocyte-derived macrophages (MDMs), PBMCs were isolated from the white blood cell fraction. The cells were suspended in RPMI/S media (RPMI 1640 media supplemented with 5% fetal bovine serum, 0.1% penicillin/streptomycin, and 10 mM L-glutamine), and the cells were plated at a density of 5 × 10⁶ cells/mL. The PBMCs were differentiated M2 macrophages in RPMI/S media supplied with 50 ng/mL recombinant human M-CSF and 50 ng/mL IL-10 (interleukin-10) for 3 days.

In Vitro NET Induction and Ultrasound Exposure
To induce NET formation, isolated neutrophils were incubated with 50 nM PMA (Sigma-Aldrich) or PBS. Thirty minutes later, cells were exposed to LIUS and incubated for 5 hours. The NETs were stained with Sytox orange (Invitrogen Corp), a nonpermeable DNA dye, for 10 minutes. To demonstrate NET clearance, NETs were stained 2 hours after PMA treatment, and M2 macrophages were stained separately with calcein AM for 30 minutes and gently scraped. Mixed cells were incubated for 30 minutes to stabilize, and then the cells were treated with LIUS for 10 minutes at 200 mW/cm² and incubated 3 hours at 37°C and 5% CO2. Live cell fluorescence images were acquired with an Axiovert 200 fluorescence microscope (Carl Zeiss). The fluorescent NET intensity was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland).

Neutrophil Elastase Activity
To quantify NET formation, the increase of NE activity in cell culture was measured using a NET assay kit according to the manufacturer’s directions (Cayman, Ann Arbor, Michigan). Twelve hours after NET induction, cells were incubated with S7 nuclease for 15 minutes to release NET-associated NE, and EDTA was added to inactivate the nuclease. Supernatants were collected and centrifuged at 300g for 5 minutes to remove cellular debris. Substrate (0.3 mM N-methoxyxysuccinyl-Ala-Ala-Pro-Val-nitroanilide) was added to supernatant and incubated for 2 hours at 37°C. Absorbance was measured at 405 nm using a microplate reader (VersaMax, Edmon ton, Canada).

Statistical Analysis
The data are expressed as the mean±95% confidence interval. The Mann-Whitney U test was used to calculate a 2-tailed P value to estimate statistical significance of differences between 2 groups. Kruskal-Wallis analysis was used to assess differences among multiple groups. All analyses were performed using IBM SPSS Statistics version 20 (IBM Corp, Armonk, New York), with a statistical significance of P<.05.

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Results
LIUS Enhanced Neutrophil Clearance and Macrophage Activation

In CFA-induced inflammatory synovium, MPO-positive neutrophils were accumulated and made a dense core after 3 days. Three days after CFA injection, the core was filled with MPO-positive and Iba1-negative cells. At 5 days postinjection, the MPO-positive neutrophils had diminished significantly and had been replaced with Iba1-positive monocytes and macrophages. The application of LIUS 1 day after induction significantly reduced MPO-positive neutrophils at 3 days (Fig. 1A), as reported previously. The LIUS-induced reduction of neutrophil accumulation might be a result of reduced infiltration or increased clearance. For this assessment, we examined synovium at 1.5 days postinduction, 12 hours after LIUS treatment. Although the degree of neutrophil accumulation at 1.5 days postinduction differed individually, LIUS did not reduce overall neutrophil accumulation. This finding suggested that LIUS induced earlier activation or the activation of more neutrophils, which were then quickly removed.

To confirm these data, MPO and Iba1 protein levels were quantified by Western blot analysis (Figs. 1B and 1C). In the CFA group that was not treated with LIUS, MPO expression peaked at 2 days postinduction and then decreased over time; although LIUS treatment tended to reduce MPO expression at all time points, the reduction was statistically significant at 3 days. In the CFA group, monocytes with Iba1 expression increased with time and peaked at day 5; however, in the LIUS-treated group, Iba1 expression peaked at day 3 and...
decreased thereafter. Taken together, these data demonstrated that MPO-positive neutrophils entered the synovial tissue, accumulated, and cleared within 5 days and that LIUS might promote neutrophil clearance of the earlier recruited and activated macrophages.

LIUS Promoted Neutrophil Apoptosis

Neutrophil clearance is central to the resolution of inflammation, which is induced mainly by apoptosis. To determine whether LIUS promotes neutrophil apoptosis, we conducted an immunohistochemical examination using TUNEL staining (Figs. 2A and B). In the CFA group, there were few TUNEL-positive cells in the dense neutrophil core at 1.5 days postinduction, after which they increased in a time-dependent manner (Figs. 2A, 2B, and 2D). When neutrophil accumulation peaked (3 days postinduction), TUNEL-positive cells exhibited a long and slender morphology, in addition to a condensed, apoptotic appearance (Fig. 2B). This morphology suggested a new type of cell death: NETosis. Treatment with LIUS increased the population of apoptotic neutrophils, which peaked at 2 days postinduction and decreased significantly at 3 days postinduction (Figs. 2A, 2B, and 2D). The LIUS-treated neutrophils exhibited earlier apoptotic changes and few apoptotic cells at 3 days postinduction, suggesting that there were few neutrophils in the synovium. A more slender, thread-like morphology and more fragmented immunoreactivity were apparent in the LIUS-treated group. The earlier appearance of apoptotic neutrophils in LIUS-treated groups may lead to their earlier removal from the synovium. However, in in vitro PMN cell cultures, TUNEL-positive neutrophils were similar in the LIUS-treated and untreated groups (Figs. 2C and 2E). These data suggest that apoptosis is involved with neutrophil clearance; however, apoptosis may comprise only part of the mechanism underlying LIUS-induced neutrophil clearance.

NET Formation Occurred in Adjuvant-Induced Synovitis and In Vitro PMN Cultures

NETosis is another type of cell death that is involved in chronic immune disorders. To observe NET formation, we examined the synovial tissue from knee joints with CFA-induced synovitis (Fig. 3). Neutrophil extracellular traps were visualized by staining for DNA (DRAQ5 [green], Hoechst [blue]), NE (red), and histone (red) in the inflamed synovium 2 days after CFA injection. Extracellular DNA was located in close proximity to neutrophils and stained positive for histone and the neutrophil granule protein NE (Fig. 3A, arrow). Neutrophil extracellular trap formation was observed in the dense core of accumulated neutrophils.
in inflamed synovium at 1.5 days after the induction of arthritis, and more NETs were observed more easily at 2 to 3 days postinduction. Low-intensity ultrasound enhanced NET formation, and more prominent NETs were observed beginning at 1.5 days postinduction (Figs. 3B and 3C). These findings suggest that NETosis is mainly involved in neutrophil clearance in CFA-induced synovitis.

We confirmed this finding in vitro PMN cultures (Fig. 4). Neutrophil extracellular traps were detected with live cell fluorescent imaging using the non-cell-permeable DNA dye Sytox orange. Neutrophil extracellular DNA exhibited long, straight processes that linked to each other to form NETs. Sytox orange-positive nuclei correspond to dying neutrophils whose DNA has not (yet) been exposed to the extracellular space. To examine the effect of LIUS on NET formation, neutrophils were treated with different intensities of LIUS. In the LIUS-treated group, we observed Sytox orange-positive nuclei, which corresponded to dying neutrophils. Low-intensity ultrasound promoted NET formation in an intensity-dependent manner (Fig. 4A). Potentiated phorbol myristate acetate is a NET-inducing agent through protein kinase C and phagocyte NADPH oxidase protein p47 (PHOX) activation and LIUS enhanced PMA-induced NET formation. Low-intensity ultrasound treatment alone induced NET formation as much as PMA treatment (Figs. 4B and 4C). However, sequential treatment with PMA and LIUS dramatically increased extracellular DNA release (Figs. 4B and 4C). During NET formation, DNA was released into the extracellular space with neutrophil granule proteins such as MPO and NE; therefore, we quantified NET release using an NE enzyme assay. Low-intensity ultrasound or PMA alone increased NE enzyme activity, and their sequential treatment synergistically increased NE activity (Fig. 4D).

LIUS Promoted NET Clearance

Macrophages clear dead neutrophils from the synovium through a process called efferocytosis. We hypothesized that resolution-phage macrophages aid in the clearance of NETs. First, we used our in vitro system to check whether macrophages clear NET DNA. We incubated M2 polarized MDMs with NET-forming neutrophils (Fig. 5A). Neutrophil extracellular traps were stained with Sytox orange (red), MDMs were stained separately with calcine AM (green), and then they were incubated together. During PMA-induced NET formation, incubation with macrophages significantly reduced extracellular DNA, and some macrophages exhibited a yellow color, which indicated the presence of DNA in the cytoplasm (Fig. 5A, images g and h). These data suggest that M2 macrophages significantly cleared extracellular DNA through phagocytosis. We observed the near-disappearance of NET DNA in the LIUS-treated group, which was quantified by measuring Sytox orange intensity (Fig. 5B). We confirmed that LIUS enhanced the clearance of protruded neutrophilic DNA. The portion of macrophages with NET phagosomes increased in the LIUS-treated group (Fig. 5C). These findings suggest that LIUS enhanced neutrophil clearance by enhancing NET formation and M2 macrophage phagocytosis.

Discussion

In the present study, LIUS promoted the process of neutrophil clearance with NETosis, which increases M2 macrophage-induced phagocytosis. Initially, earlier clearance of neutrophils can block the following neutrophil infiltration because neutrophils are able to recruit additional neutrophils into the inflammatory core. The clearance of neutrophils might be involved with increased neutrophilic cell death, which was related to apoptosis and NETosis. In the present study, LIUS induced NETosis of neutrophils, which might help boost neutrophil clearance during the resolution of inflammation (Fig. 6).

The role of inflammation in synovitis has diverse effects on the modulation of networks. During synovitis, proinflammatory mediators (eg, IL-1, tumor necrosis...
factor, and IL-6) and anti-inflammatory mediators (eg, IL-4, IL-13, and tissue inhibitors of metalloproteinases) have beneficial or detrimental effects on the progression of arthritis.\textsuperscript{17–19} In inflamed synovium, the innate immune cells play important roles in the induction, maintenance, and progression of arthritis. The infiltration of neutrophils into the inflamed synovium is prominent 3 days after inflammation induction and features a neutrophil core surrounded by Iba-1-positive monocytes. In our study, we began to apply LIUS on CFA-induced synovitis at 1 day postinduction. Low-intensity ultrasound showed the decreased accumulation of neutrophils and monocytes after 3 days; however, LIUS did not reduce neutrophil accumulation in earlier phase (1.5 days). The protective effects of LIUS on synovitis exhibited increased neutrophil clearance during the resolution phase, rather than decreased neutrophil infiltration itself. The effect of LIUS on the induction of inflammation must be investigated further.

Neutrophil homeostasis is critical; an imbalance can produce inappropriate inflammatory states.\textsuperscript{33} Dead neutrophils can propagate the inflammatory response, in turn leading to tissue destruction; conversely, the clearance of dying neutrophils is critical to the resolution of inflammation. Therefore, neutrophils should be considered important targets for the development of new therapies. Drugs already used to manage RA downregulate neutrophilic functions, including migration to the joint, degranulation, and the production of inflammatory mediators.\textsuperscript{34}

The modulation of neutrophil clearance also is an important arthritis-modifying target. Cytokine withdrawal, as it occurs during the resolution phase of inflammation, leads to the induction of apoptosis.\textsuperscript{35} The apoptosis of neutrophils is a well-regulated mechanism for keeping cellular homeostasis under physiological

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**Figure 4.**
In vitro low-intensity ultrasound (LIUS)–induced neutrophil extracellular trap (NET) formation in polymorphonuclear (PMN) cell culture. Using the non–cell-permeable DNA dye Sytox orange (red) in cultured PMN cells, NETs were detected with live cell fluorescent images 5 hours after LIUS treatment. (A) LIUS increased NET formation in an intensity-dependent manner. Polymorphonuclear cells were treated with different intensities of LIUS: 0 mW/cm\textsuperscript{2} (a), 50 mW/cm\textsuperscript{2} (b), 100 mW/cm\textsuperscript{2} (c), and 200 mW/cm\textsuperscript{2} (d). These images are representative of 3 independent experiments (n=3). Scale bars=200 \textmu m. (B) Low-intensity ultrasound enhanced phorbol myristate acetate (PMA)–induced NET formation. Neutrophil extracellular traps were stained with Sytox orange in control (a and e), 200 mW/cm\textsuperscript{2} of LIUS (b and f), PMA (c and g), and sequential treatment of PMA and LIUS (d and h). These images are representative of 3 independent experiments (n=3). Scale bars: a–d=200 \textmu m, e–h=100 \textmu m. (C) Quantification of NET formation from 6 different images for each group. The intensity of Sytox orange was measured using the Image J program. Data are displayed as means±95% confidence intervals. *P<.05 vs control, †P<.05 vs LIUS, and ‡P<.05 vs PMA. (D) Neutrophil elastase (NE) activity was measured at 12 hours after NET induction. Media were removed, and NETs were digested with S7 nuclease to measure NET-associated NE activity. Data are displayed as means±95% confidence intervals. *P<.05 vs control, †P<.05 vs LIUS, and ‡P<.05 vs PMA. US = ultrasound.
conditions. Delayed apoptosis is one important mechanism underlying neutrophil accumulation. Synovial leukocyte apoptosis is inhibited in established RA; in contrast, high levels of leukocyte apoptosis are observed in self-limiting arthritis. In our study, although LIUS tended to increase the proportion of TUNEL-positive cells in the core of neutrophils, the rate of apoptotic neutrophil death in the core was low compared with the total population of neutrophils. Therefore, we hypothesize that there is another mechanism for neutrophil clearance in CFA-induced synovitis.

Upon activation, neutrophils release DNA and a subset of their granule content to form NETs. Neutrophil extracellular traps participate in the pathogenesis of autoimmune and inflammatory disorders, with proposed involvement in RA, glomerulonephritis, chronic lung disease, sepsis, and vascular disorders. Exaggerated NETosis or diminished NET clearance likely increases the risk of autoreactivity to NET components. The biological significance of NETs is just beginning to be explored. The present study revealed that NETs are involved with adjuvant-induced synovitis, and LIUS enhances NET formation and can assist NET clearance with phagocytic macrophages. We confirmed the effect of LIUS on NETs and neutrophil clearance with M2 macrophages in in vitro peripheral neutrophil cultures. Therefore, it is possible that NETs act during the resolution of the inflammatory response and finally result in neutrophil clearance by enhancing the phagocytosis of macrophages. Low-intensity ultrasound significantly increases NET formation in the core of neutrophil accumulation compared with the control group. It is feasible that LIUS-induced NETs are involved in more and earlier neutrophil clearance, with increased phagocytosis of macrophages (Fig. 5).

Figure 5.
Low-intensity ultrasound (LIUS) promoted neutrophil extracellular trap (NET) clearance. (A) Low-intensity ultrasound promoted NET clearance by enhancing macrophage phagocytosis. Neutrophils were treated with 30-nM PMA for 2 hours to induce NET clearance compared with the NETs stained with Sytox orange (red). The M2 macrophages (calcein AM [green]) were stained separately, and macrophages were added to NET-stained neutrophils. Cells were treated with LIUS 30 minutes after macrophage addition, and images were obtained 3 hours after LIUS treatment. These images are representatives of 3 independent experiments (n = 3 animals per experiment). Scale bar = 100 μm. (B) Remaining NETs were quantified from 6 different images for each group. The intensity of Sytox orange was measured using the Image J program. Data are displayed as means ± 95% confidence intervals. *P < .001 vs control, #P < .001 vs phorbol myristate acetate (PMA), and †P < .05 vs PMA + M2. (C) The NET-merged macrophages were counted and normalized by total M2 macrophages from 5 different images for each group. Data are displayed as means ± 95% confidence intervals. †P < .001 vs PMA + M2, US = ultrasound.
This study had some limitations. First, the effect of LIUS on synovial inflammation was observed only during the acute phase, although the CFA synovitis model is known as a chronic or subacute animal model. Second, in vivo NETosis cannot be quantified, although it has been demonstrated to occur more in LIUS-treated rats than in nontreated rats. However, the effect of LIUS on NETosis in the in vitro neutrophil culture system was clear, with limitations of artificial conditions, including enhanced fluid flow and shear forces. The energy introduced by LIUS cannot be measured accurately in the relatively small tissue volume of this animal model.

In summary, synovial inflammation can be an important element of the pathogenesis of progressive arthritis. In our study, CFA-induced synovitis exhibited synovial hyperplasia with neutrophil infiltration followed by monocytes and macrophages. Low-intensity ultrasound promoted neutrophil clearance with NETosis, a new type of neutrophil death. Increased neutrophil clearance during the resolution of inflammation may be suggested as a therapeutic mechanism of LIUS.

Figure 6.
Protective mechanism of low-intensity ultrasound (LIUS) on synovitis. In the initiation of synovitis, massive neutrophils are recruited into the synovium. The neutrophils release granules to remove microbes or infected tissues. Some neutrophils have died due to apoptosis or neutrophil extracellular trap (NETosis), which can activate macrophages. Removal of dying neutrophils by M2 macrophages is the first step of resolution. Low-intensity ultrasound promoted the process of neutrophil clearance with earlier apoptosis of neutrophils and enhanced NETosis, which increases M2 macrophage-induced phagocytosis. The earlier clearance of neutrophils can block the following neutrophil infiltration.

All authors provided concept/idea/research design. Dr. Chung and Dr. Baik provided writing, data collection and analysis, and consultation (including review of manuscript before submission). Dr. Baik provided project management, fund procurement, participants, facilities/equipment, and institutional liaisons.

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References


**eFigure.**
Schematic illustration of low-intensity ultrasound (LIUS) exposure systems in vivo and in vitro. (A) The LIUS probe was attached to anterior left knee joint, where ultrasound transmission gel was applied to minimize reflection. (B) Culture dishes were placed on the probe in which the ultrasonic beam was confined, within the effective radiating area of the transducer. The gap between the probe and polystyrene dish was filled with ultrasound transmission gel, and LIUS penetrated the polystyrene dish and reached the neutrophils.